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14. ABSTRACT Rheumatoid arthritis (RA) and multiple sclerosis (MS) are chronic inflammatory autoimmune diseases affecting millions of people. Here we are proposing a novel approach to cure MS, by administration of a specific strain of human commensal-bacteria. Recent studies have shown that intestinal microflora plays an important role in the health of the host and possesses probiotics like qualities. We hypothesize that Gram-negative commensal bacteria from human gut have the potential to be used as a therapeutic agent. We have used collagen induced arthritis (CIA) in HLA-DR4DQ8 mice and PLP91-110 induced experimental autoimmune encephalomyelitis (EAE) HLA-DR3DQ8 mice to test our hypothesis that treatment with commensal bacteria <i>Prevotella histicola</i> can modulate disease. First using various doses of bacteria, we have identified the optimal dose to be used for treatment of CIA as well as EAE. Treatment of mice with <i>P. histicola</i> as probiotics is ongoing. Our study showed that treatment of mice with 3-4 doses of <i>P. histicola</i> in collagen/PLP91-110-immunized mice led to suppression of antigen-specific immune response in-vitro in both CIA as well as EAE model. Our data indicates that <i>P. histicola</i> induced immune responses in the gut cause induction of immune tolerance in periphery leading to suppression of antigen specific response.					
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This progress report is from April '2010 to Mar '2011

Introduction

Rheumatoid arthritis (RA) and multiple sclerosis (MS) are chronic inflammatory autoimmune diseases affecting millions of people. Since these diseases occur more often among young and middle-aged adults, they cause significant loss of productive years in this population. Beside these autoimmune diseases also cause significant economic burden (hundreds of Billions of USD) on society. Although several therapies are in use, none of them cure disease. In this study we are investigating a unique approach to ameliorate RA and MS, by administration of a specific strain of human commensal bacteria *Prevotella histicola* (*P histicola*), which was recently isolated from human gut. Among all the genetic factors linked with RA and MS, the strongest association has been with the MHC class II region on chromosome 6 (1) and we have generated novel humanized HLA class II transgenic animal models of RA and MS. We are utilizing these animal models to test the therapeutic efficacy of *P histicola*. Using an experimental autoimmune encephalomyelitis (EAE), which is an animal model of MS, we showed that HLA-DR3DQ8 transgenic mice develop MS like disease characterized by brain plaques (2). HLA-DR3DQ8 mice were immunized with CNS antigen PLP91-110 and received either *Prevotella histicola* or medium starting day 7 post-immunization and every other day for a total of 7 doses. Mice were followed for weight loss, disease incidence, duration and severity for 4 weeks post-immunization. Our study showed that treatment of mice with 3-4 doses of *P. histicola* in PLP-immunized mice led to suppression of antigen-specific immune response *in vitro*. Treatment with *P histicola* cause suppression of inflammatory cytokine IL-17 and increase in levels of anti-inflammatory cytokine IL-10. Further, we observed that HLA-DR3DQ8 mice treated with *P histicola* led to lower clinical disease incidence as well as severity indicating immunosuppressive properties of *P histicola*. First using various doses of bacteria, we have identified the optimal dose to be used for treatment of EAE in HLA-DR3DQ8 transgenic mice. Treatment of mice with *P histicola* as probiotics is ongoing in EAE model. Similarly, we studied therapeutic efficacy of *P histicola* to modulate arthritis in murine model of RA known as collagen induced arthritis (CIA) (3). Our *in vitro* studies showed that treatment of mice with *P. histicola* in collagen-immunized mice led to suppression of antigen-specific immune response and reduction in production of inflammatory cytokines. We have standardized the optimal therapeutic dose of *P. histicola* for mice and then used that dose for treatment of arthritis. Treatment of mice with *P. histicola* as probiotics and therapy are ongoing. Thus in summary, we have made good progress in 1st year and our data suggests that *P histicola* induced immune responses in the gut might cause induction of immune tolerance in periphery leading to suppression of antigen specific response.

We have performed following tasks-

Progress report (arranged according to approved SOW)



Fig-1 Anaerobe Laked Sheep Blood Agar Plate streaked with *P. histicola*.

SOW 1-a) Bacterial isolation and culture time frame (1-3 months)

An isolate of *Prevotella histicola*, stored at -70°C in skim milk, was inoculated onto a CDC Anaerobe Laked Sheep Blood Agar with Kanamycin and Vancomycin (KV) (Becton, Dickson and Company, Sparks, MD) and incubated anaerobically in an anaerobic jar with AnaeroPack® system (Mitsubishi Gas Chemical America, Inc., New York, NY) and incubated at 37°C for 2-3 days. The bacterium was then swabbed into 10 mL of Trypticase soy broth and is anaerobically incubated for 2 days. We have grown sufficient quantity of *P. histicola* for our experiments.

Milestone #1 *P. histicola* was isolated from a patient and cultured. The culture were built up to use it for both models.

SOW 1-b) Prepare forms for approval of Animals use and protocols involved (1-2 months)

Milestone #2 Animal use approvals was done before start of the grant as per requirement.

SOW 1-c) Generation of transgenic mice for *in vivo* studies (4-8 months)

DR4DQ8 transgenic mice Dr. Taneja

Characterization of transgenic mice: During this phase, we generated transgenic mice expressing HLA-DR4 and DQ8 and extended our studies to double transgenic mice. First, all transgenic mice used *in vivo* and *in vitro* experiments were characterized for the presence of HLA genes by flow cytometry (Fig 2A). Only mice positive for the transgene were used for experiments.

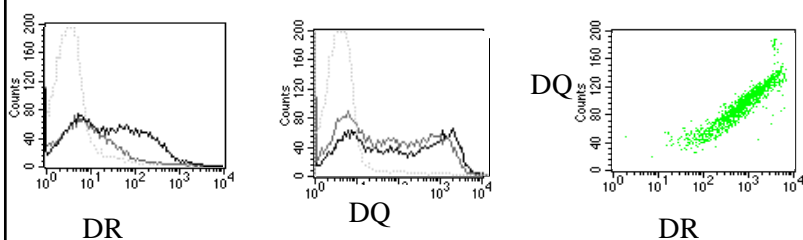


Fig 2A. Expression of HLA-DR4 and DQ8 in transgenic mice was analyzed by Flow cytometry in cells isolated from spleen after staining with conjugated antibodies. Expression of DR4 and DQ8 of AE⁰.DRB1*0401.DQ8 mice shows more number of cells expressing the DR and DQ transgene compared to single transgenic mice, Gray line- DRB1*0401.DQ8, Black line- DRB1*0401 mice in DR (left) and DQ8 mice in DQ (middle). Right, B cells express both DQ and DR on the same cell.

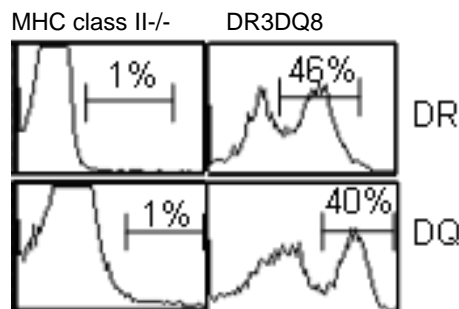


Fig 2 B: Representative FACS histogram showing HLA-DR and DQ expression on splenocytes in HLA-DR3DQ8 double transgenic mice. Splenocytes were analyzed for cell surface markers HLA-DR/HLA-DQ by flow cytometry. Numbers in histograms indicate the percentage of cells positive for the HLA-DR/HLA-DQ marker.

DR3DQ8 transgenic mice Dr. Mangalam: Similar to DR4DQ8 transgenic mice, we also generated DR3DQ8 transgenic mice to be used for EAE studies. All mice were stained for presence of DR3 and DQ8 using HLA-DR/DQ specific antibodies and analyzed by flow cytometry. Fig.2B is a representative diagram showing expression profile of DR3DQ8 mouse expressing both HLA-DR and –DQ molecule. Only mouse expressing both –DR3 as well as DQ8 molecules were used for the studies.

Milestone # 3 Transgenes in mice can segregate which necessitates various matings to have enough mice for in vivo work.

SOW 1-d) Feeding bacteria in preventive protocol

CIA model (9-15th months) Dr. Taneja

Modulation of arthritis in preventive protocol: We immunized mice with type II collagen (CII) to induce arthritis and then fed them *P. histicola* on alternate days 2 weeks following immunization. Next we tested mice in preventive protocol. Mice were fed *P. histicola* 12 days prior to immunization with type II collagen. Mice immunized with type II collagen and fed media without bacteria as well as mice fed bacteria without CII immunization were used as controls. Disease phenotype in CIA model is characterized by paw swelling, scored on scale of 0-3. The studies are in progress and mice are being monitored for development of arthritis. Sera and paws will be collected at the termination of experiments.

EAE model (9-15th months) Dr. Mangalam

Modulation of EAE by *P. histicola*- Standardization of frequency for optimum therapeutic effect
EAE was induced in HLA-DR3DQ8 transgenic mice by immunization with proteolipid protein (PLP) peptide 91-110 emulsified in CFA at 1:1 ratio. These mice also received pertussis toxin at day 0 and day 2 post-immunization. Immunized mice were treated with bacteria (oral gavage) 7 day post immunization. DR3DQ8 mice received either *Prevotella histicola* or medium starting day 7 post-immunization and every other day or every 3rd day for a total of 7 doses. Mice were followed for weight loss, disease incidence, duration and severity for 4 weeks post-immunization. We have observed similar suppressive activity in both protocols, i.e. that feeding *P. histicola* every 2nd day or every 3rd day had same beneficial effect.

Modulation of EAE by *P. histicola* in preventive protocol

Next we tested mice in preventive protocol. Mice were fed either with *P. histicola* or medium for 12 days prior to immunization with PLP91-110. Mice were observed daily for clinical symptoms and disease severity was scored as follows: 0, normal; 1, loss of tail tone; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis and forelimb paralysis or weakness; 5, moribundity/death. Mice of both sexes were used. The studies are in progress and mice are being monitored for development of EAE. Brain, spinal cord and sera will be collected at the termination of experiments.

SOW 1-e) Immunization of mice with relevant antigen and feeding bacteria in ongoing disease In vivo (11-12th months)

CIA 20 mice Controls 20	Dr. Taneja	Ongoing
EAE 20 mice Controls 20	Dr. Mangalam	Ongoing

SOW 1-f) Score immunized mice for arthritis in CIA model. Bleed mice via tail technique (11-16th months) Dr. Taneja. **Ongoing**

Score immunized mice for paralysis in EAE model. Bleed mice via tail technique (11-16th months) Dr. Mangalam **Ongoing**

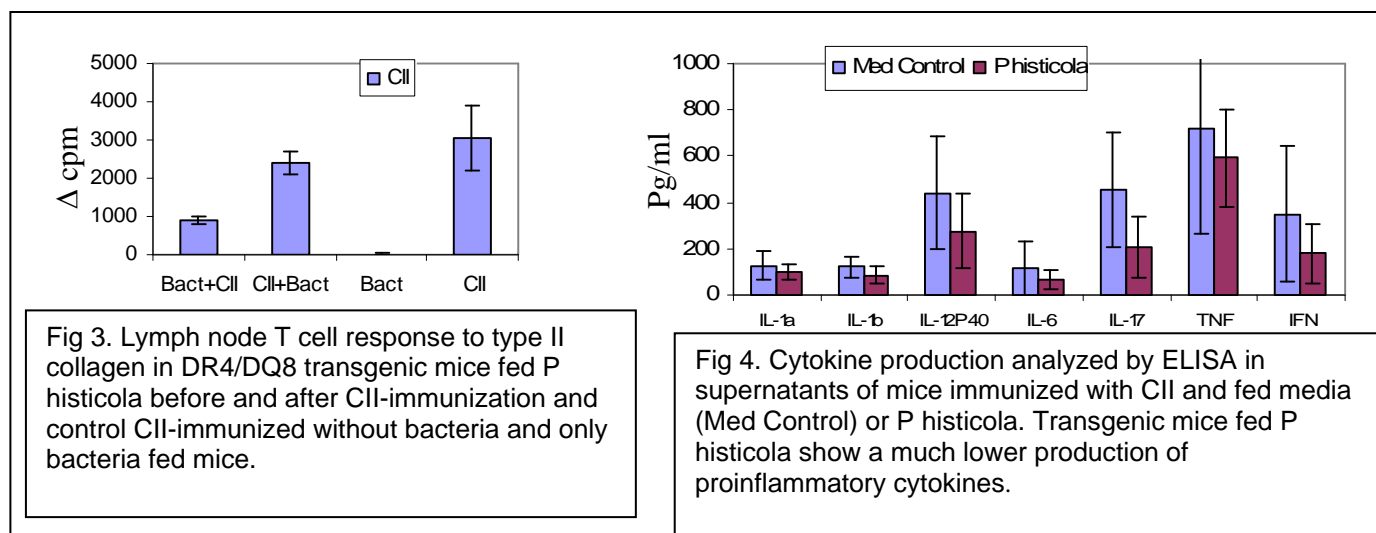
SOW 1-g) Sacrifice CIA group mice and harvest various organs and snap freeze a part of organs and one part for paraffin blocks (16-17th months) Dr. Taneja **Ongoing**

Sacrifice EAE mice and harvest various organs and snap freeze a part of organs and one part for paraffin blocks (16-17th months) Dr. Mangalam **Ongoing**

SOW 1-h) Harvest spleen and lymph nodes from the treated and controls mice in CIA (arthritis) model and do *in vitro* assay for T cell response to antigen and measure cytokines (17 month) Dr. Taneja

CIA (arthritis) model (20th month). Dr. Taneja

Prevotella histicola modulates antigens-specific responses: Since collagen specific T-cell responses play an important role in disease pathogenesis of CIA, we investigated effect of *Prevotella* on antigen specific immune response and production of pro-inflammatory cytokines by antigen specific T-cells. Mice were fed bacteria before or after immunization with CII. Mice immunized with but no bacteria and mice fed bacteria without CII-immunization were used as controls. As shown below in Fig 3, antigen-specific T cell response was suppressed in mice fed *P. histicola* before and after immunization with CII as compared to mice immunized with CII only. As expected, mice fed bacteria in the absence of CII-immunization did not show any antigen specific response. We further tested and compared production of pro-inflammatory cytokines in mice immunized with CII and fed medium and mice immunized with CII and fed *P histicola* (Fig 4). Mice treated with bacteria after CII-immunization showed a much lower production of proinflammatory Th1 (IL-1, TNF and IFN) as well as Th17 (IL-12(p40), IL-17, IL-6) cytokines compared to mice immunized with CII and fed media without bacteria. These *in vitro* studies clearly show an immunomodulatory role of commensal bacteria like *P histicola*. Our studies suggest that *P. histicola* may be able to generate systemic suppression via mucosal immune regulation.



SOW 1-h) Harvest spleen and lymph nodes from the treated and controls mice in EAE (multiple sclerosis) model and do *in vitro* assay for T cell response to antigen and measure cytokines (17 month) Dr. Mangalam

EAE (MS) model (20th month). Dr. Mangalam

Modulation of antigen specific T cell and cytokine response by *P. histicola*

To determine if this protective effect of *P. histicola* is due to own-regulation of antigen specific T cell responses, we isolated splenocytes from mice treated with bacteria or medium and stimulated with PLP₉₁₋₁₁₀ peptide. As shown in Fig. 5, antigen specific T cell response was suppressed in DR3DQ8 mice treated *P. histicola* as compared to sham treated mice. Splenocytes from bacteria fed mice also produce less pro-inflammatory cytokine IL-17 on stimulation with PLP, while levels of anti-inflammatory cytokine IL-10 was increased (Fig. 6). Levels of IFN- γ were not significantly different between two groups (Fig. 6).

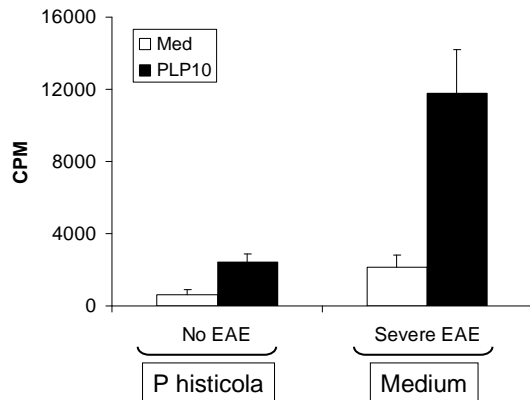


Fig. 5. *P. histicola* treated DR3DQ8 mice exhibited reduced PLP₉₁₋₁₁₀ specific T cell proliferation as compared to sham treated mice. Splenocytes were collected from mice immunized with PLP and treated with *P. histicola* or medium (sham) and were stimulated *in vitro* with the PLP₉₁₋₁₁₀ polypeptide.

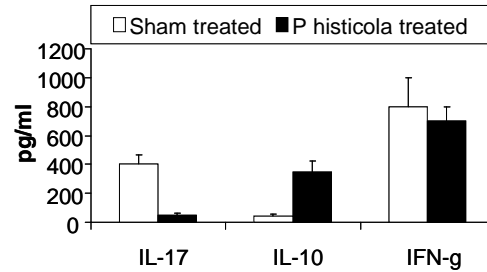


Fig.6 - *P. histicola* treated DR3DQ8 mice exhibited reduced level of IL-17 and increased levels of IL-10 as compared to sham treated mice. Levels of IFN- γ were not different

The Key Research Accomplishments

- Culture of *P. Histicola* for use in both CIA and EAE model
- Generation of DR4/DQ8 transgenic mice for in vivo use. HLA-DR4 and HLA-DQ8 transgenic mice are mated to generate double transgenic mice. Double transgenic mice are characterized for the presence of HLA transgenes by flow cytometry using specific conjugated antibodies. Mice positive for both genes are identified and mated. DR4 and DQ8 transgenes can segregate which necessitates typing for the transgene positivity.
- Generation of DR3/DQ8 transgenic mice for in vivo use. HLA-DR3 and HLA-DQ8 transgenic mice were mated to generate double transgenic mice. Double transgenic mice are characterized for the presence of HLA- DR3 and –DQ8 transgenes by flow cytometry using specific conjugated antibodies. Mice positive for both genes are identified and mated. DR3 and DQ8 transgenes can segregate which necessitates typing for the transgene positivity.
- Mice were gavaged with *P. histicola* for 2 weeks and then immunized with either type II collagen (DR4DQ8 mice) PLP₉₁₋₁₁₀ peptide (DR3DQ8 mice). In addition, control mice were gavaged with media in which *P. histicola* were cultured and immunized with type II collagen or PLP₉₁₋₁₁₀ peptide. Mice are being monitored for disease (CIA in DR4DQ8 and EAE in DR3DQ8).
- Sera from all test and control mice is being collected and will be used to study antibodies at the termination of the experiment.
- In vitro experiments show that feeding bacteria suppressed antigen-specific T cell response and reduced production of inflammatory cytokines in both CIA and EAE models.

Reportable Outcome

We have presented two abstracts based on above findings at meeting titled **Microbiota and mucosal immunology: the interface in health and disease**, April 14-16, 2011, San Francisco, CA, USA

Abstracts:

- David Luckey, Melissa Karau, Robin Patel, Moses Rodriguez, Joseph Murray, Chella David, Veena Taneja and Ashutosh Mangalam (2011). Human commensal bacteria as a novel therapeutic agent for Multiple Sclerosis. Microbial and Mucosal Immunology: the interface in health and disease, San Francisco, CA, USA.
- David Luckey, Marshall Behrens, Melissa Karau, Robin Patel, Ashutosh Mangalam, and Veena Taneja (2011). Microbial Mucosal Modulation of Arthritis. Microbial and Mucosal Immunology: the interface in health and disease, San Francisco, CA, USA.

Conclusions. Our in vitro data showing suppression of antigen-specific immune response in *P. histicola* treated mice suggesting generation of peripheral tolerance via gut. The results from our ongoing in vivo experiments will help us in understanding if *Prevotella histicola* can be used as a treatment of EAE in HLA-DR3DQ8 transgenic mice and CIA in DR4DQ8 mice.

References

1. Baranzini, S. E., J. Wang, R. A. Gibson, N. Galwey, Y. Naegelin, F. Barkhof, E. W. Radue, R. L. Lindberg, B. M. Uitdehaag, M. R. Johnson, A. Angelakopoulou, L. Hall, J. C. Richardson, R. K. Prinjha, A. Gass, J. J. Geurts, J. Kragt, M. Sombekke, H. Vrenken, P. Qualley, R. R. Lincoln, R. Gomez, S. J. Caillier, M. F. George, H. Mousavi, R. Guerrero, D. T. Okuda, B. A. Cree, A. J. Green, E. Waubant, D. S. Goodin, D. Pelletier, P. M. Matthews, S. L. Hauser, L. Kappos, C. H. Polman, and J. R. Oksenberg. 2009. Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis. *Human molecular genetics* 18:767-778.
2. Mangalam, A., D. Luckey, E. Basal, M. Jackson, M. Smart, M. Rodriguez, and C. David. 2009. HLA-DQ8 (DQB1*0302)-restricted Th17 cells exacerbate experimental autoimmune encephalomyelitis in HLA-DR3-transgenic mice. *J Immunol* 182:5131-5139.
3. Taneja, V., and C. S. David. Role of HLA class II genes in susceptibility/resistance to inflammatory arthritis: studies with humanized mice. *Immunological reviews* 233:62-78.
4. David Luckey, Melissa Karau Robin Patel, Moses Rodriguez, Joseph Murray, Chella David, Veena Taneja and Ashutosh Mangalam. "Human commensal bacteria as a novel therapeutic agent for Multiple Sclerosis" for meeting titled Microbiota and mucosal immunology: the interface in health and disease, April 14-16, 2011, San Francisco, CA, USA
5. David Luckey, Marshall Behrens, Melissa Karau, Robin Patel, Ashutosh Mangalam, and Veena Taneja (2011). Microbial Mucosal Modulation of Arthritis. Microbial and Mucosal Immunology: the interface in health and disease, San Francisco, CA, USA.

Appendices (2 abstracts)

Abstract submitted to Microbiota and mucosal immunology: the interface in health and disease, April 14-16, 2011, San Francisco, CA, USA

1. Human commensal bacteria as a novel therapeutic agent for Multiple Sclerosis

David Luckey¹, Melissa Karau², Robin Patel², Moses Rodriguez^{1,3}, Joseph Murray⁴, Chella David¹, Veena Taneja¹ and **Ashutosh Mangalam¹**.

Department of Immunology, Clinical Microbiology, Neurology, and Gastroenterology, Mayo Clinic, Rochester, MN -55905 USA.

Multiple sclerosis (MS), a chronic inflammatory disease of the CNS, is strongly associated with the MHC class-II genes HLA-DR2, DR3, DR4, DQ8. Here we are proposing a novel approach to cure MS, by administration of a specific strain of human commensal-bacteria. Recent studies have shown that intestinal microflora plays an important role in the health of the host and possesses probiotics like qualities. We hypothesize that Gram-negative commensal-bacteria *Prevotella histicola* from human gut have the potential to be used as a therapeutic agent. We have used HLA-DR3DQ8 transgenic mice to test our hypothesis that treatment with commensal-bacteria *P. histicola* can modulate experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Previously we showed that PLP₉₁₋₁₁₀ can induce EAE in HLA-DR3DQ8 transgenic mice. First using various doses of bacteria, we have identified the optimal dose to be used for treatment of EAE. Our study showed that treatment of mice with 3-4 doses of *P. histicola* in PLP₉₁₋₁₁₀-immunized mice led to suppression of antigen-specific immune response *in-vitro*. Treatment of mice with *P. histicola* as probiotics is ongoing. Our data indicates that *P. histicola* induced immune responses in the gut cause induction of immune tolerance in periphery leading to suppression of antigen-specific response.

2. Microbial mucosal modulation of arthritis

David Luckey, Marshall Behrens, Melissa Karou, Robin Patel, Joseph Murray, Ashutosh Mangalam and Veena Taneja

Department of Immunology, Gastroenterology and Microbiology, Mayo Clinic Rochester, MN -55901 USA.

Predisposition to rheumatoid arthritis (RA) is associated with the presence of genetic factors, HLA class II molecules, DR4 and DQ8, being the strongest. Recent reports that patients with RA have decreased fecal levels of certain commensal bacteria suggested that intestinal microbes might be critical in regulation of disease. We isolated *Prevotella histicola*, anaerobic commensal bacteria of Human gut, from bowel of a patient and have shown that it possesses anti-inflammatory activity. We propose that gut microbiota can influence peripheral immune response and may modulate arthritis in a murine model. We have established a murine model of rheumatoid arthritis using mice expressing RA-associated HLA genes, DRB1*0401 and DQ8. DR4 and DQ8 mice develop collagen-induced arthritis (CIA) following immunization with type II collagen (CII). We have used HLA-DR4/ DQ8 mice to test our hypothesis that treatment with commensal bacteria like *Prevotella histicola* can modulate CIA. In vitro data showed that treatment of mice with *P. histicola* in CII-immunized mice led to suppression of antigen-specific immune response and reduction in production of inflammatory cytokines suggesting *P. histicola* has anti-inflammatory properties in this model. Treatment of CIA in transgenic mice in therapeutic protocol is ongoing. Our data suggests that *P. histicola* induced immune responses in the gut causes systemic immune suppression and may be able to regulate autoimmunity.